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14. ABSTRACT: Human (hFOB 1.19) and murine (MC3T3-E1) osteoblasts grew for extended periods (up to 10 months) in a specialized bioreactor. Over time the cells formed multilayers and a collagenous matrix with mineralized nodules and small chips of "bone". The number of cell layers in the bone-like tissue peaked at about 30 to 60 days and then declined. This change was reflected in the cell morphology. With time, the osteoblasts transitioned from multilayer cuboidal cells to flat osteocyte-like cells. We characterized the response of MC3T3-E1 bone-like tissue at various stages of maturity to metastatic human MDA-MB-231 breast cancer cells using a variety of approaches. The cancer cells attached, grew, and penetrated the matrix. Within 2 days of co-culture, the cancer cells replicated and organized into linear files. Close inspection of both 2D optical sections and 3D reconstructions revealed concomitant remodeling of the tissue. Over 3 days of co-culture, cuboidal osteoblasts became elongated and aligned themselves with cancer cells. The osteoblasts responded with production of IL-6, a characteristic osteoblast inflammatory stress cytokine. These data support our idea that the bioreactor will serve as a useful in vitro model to study the interaction of metastatic breast cancer cells and osteoblasts.					
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INTRODUCTION:

Breast cancer frequently metastasizes to bone and disrupts the homeostatic balance between osteoblast (bone forming) and osteoclast (bone degrading) cells, ultimately leading to osteolytic degradation of bone tissue. The objective of this study was to test the hypothesis that osteolytic bone degradation results not only from the well-known stimulation of osteoclasts but also from a heretofore-unrealized affect of the cancer cells on osteoblasts. Specifically, we hypothesized that breast cancer cells inhibit osteoblastic accretion of mineralized tissue, which accelerates skeletal degradation and prevents healing of bone lesions. In order to test this idea, we proposed to develop an existing three-dimensional culture system (hereafter termed bioreactor) into an *in vitro* test system for studying the interactions between osteoblasts and metastatic breast cancer cells. The objectives were to characterize the morphology and physiology of osteoblasts (MC3T3-E1) cultured in bioreactors to produce a 3 D matrix and to determine how the osteoblasts reacted to the presence of human metastatic breast cancer cells (MDA-MB-231).

BODY:

Task 1. To determine the effects of metastatic breast cancer cells on the physiology of osteoblasts cultured in a long-term culture system that fosters growth in three-dimensions. (Months 1-6 of proposed work.)

Establish cultures of MC3T3-E1 cells in bioreactors and add metastatic breast cancer cells at various times after the establishment of culture (4,7,15,30 days). Periodically sample the secreted materials in the growth chamber that will indicate osteoblast function. ELISA or RIA will be carried out for OCN, IL-6, MIP-2, and MCP-1. Alkaline phosphatase will be assayed by a biochemical assay. Culture medium from cells grown in standard tissue culture will be compared. For control cells in selected assays, a human immortalized non-tumorigenic cell line, such as h-TERT–HME1 will be used. Establish cultures of MC3T3-E1 cells in bioreactors and add metastatic breast cancer cells at various times after the establishment of culture bioreactors as in task 1-a. Terminate the cultures periodically to assay the cells for cell associated alkaline phosphatase, Type I collagen, mineralization (alizarin red, von Kossa) and for apoptosis (TUNEL).

Task 2. To determine the effects of metastatic breast cancer cells on osteoblast morphology in a long-term bioreactor culture system that fosters growth in 3-dimensions. (Months 7-13 of proposed work.)

Osteoblasts and breast cancer cell co-cultures will be prepared as in Task 1. The stage of differentiation of the osteoblasts and the time of the addition of the cancer cells will be

decided based on the results of Task 1. Co-cultures from the bioreactor and conventional cultures will be fixed to preserve morphological detail. Part of each culture will be fixed with paraformaldehyde following a protocol to preserve GFP. These cultures will be imaged with confocal fluorescence microscopy to detect the metastatic breast cancer cell migration. Part of each culture will be prepared for detection of apoptosis (TUNEL). The GFP tag of the cancer cells will allow us to distinguish apoptotic cancer cells from apoptotic osteoblasts. Part of each culture will be prepared for scanning electron microscopic observation. We anticipate that we will be able to distinguish cancer cells from osteoblasts in these preparations based on size and shape. Part of each culture will be prepared for the transmission electron microscopy. We will view the cells with an eye to fine structural detail.

Summary of Results of First Year Investigation

Tasks 1 and 2 were pursued in parallel to maximize efficiency in achieving aims of proposed work and to provide internal consistency in the work by using living cells/biological materials in a conserved timeframe.

Characterization of Bioreactor Culture of Osteoblasts:

Main finding: Osteoblast lines grew into bone-like tissue in the bioreactor.

We had previously demonstrated that human (hFOB1.19) and murine (MC3T3-E1) osteoblasts could be maintained in a “Bioreactor” culture system originally developed by Vogler^{1,2} (termed “bioreactor” in the following). We continued to characterize these two cell lines in the system and published the results³ (publication appended). The significant finding was that bioreactor culture permitted long-term (4 month) culture of osteoblasts with apparently normal phenotype and development of a matrix secreted by the cells comprised of 4-6 cell layers (Fig. 1). The cells expressed bone alkaline phosphatase activity, stained positive for mineralization as determined by alizarin red and von Kossa staining. The MC3T3-E1 derived matrix exhibited especially high levels of mineralization. These results were contrasted to culture of identical cells using conventional tissue culture methods. Osteoblast “tissue” grown using conventional methods was comprised of fewer cell layers and cells exhibited twice the rate of apoptosis after only 30 days in continuous culture.³ We

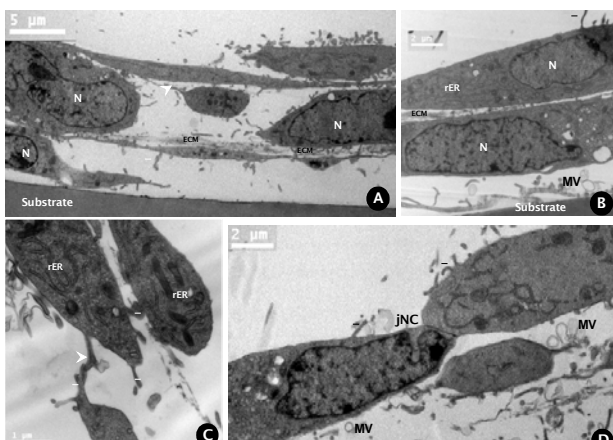


Fig.1. Ultrastructure of hFOB 1.19-derived tissue (15 d) recovered from the bioreactor by cross-sectional TEM. Arrows point to cell protrusions that occasionally connect two cells, as shown in Panel C. Annotations: jNC = gap junction; MV = matrix vesicles; N = nucleus; rER = rough endoplasmic reticulum.

concluded that conventional culture was inappropriate for long-term maintenance of bone-like tissue produced *in vitro*.

Following this published study, we found that MC3T3-E1 derived bone can be maintained for indefinite culture periods. In fact, we have demonstrated that this tissue can be maintained for 10 months of continuous culture, to our knowledge a record achievement in tissue culture/tissue engineering. And there were no indications that this 10-month tissue was at all stressed, suggesting that the culture would have been viable for many more months. In fact, after about 8 months of culture, macroscopic bone became visually apparent, culminating in 10 months with formation of a white, brittle material that exhibited an orange-peel texture that proved to be nearly identical to bovine bone by x-ray scattering (Fig. 2). Formation of macroscopic bone *in vitro* is, to our knowledge, another first in bone-cell tissue culture. This macroscopic bone was especially evident in the form of deposits on the dialysis membrane (Fig. 3C). The exact relationship among matrix vesicles (Fig. 1), mineralized matrix (Fig. 2), and bone chips (Fig. 3) are unclear at this time, but we speculate that contents of the many matrix vesicles observed in mineralizing osteoid contributes to the nucleation of extracellular mineral that templates in sheet-form onto the dialysis membrane. Such a process would be consistent with intramembranous bone formation, e.g. skull or flat bones, or with the remodeling of long bones. Clearly, more detailed investigation is required to fully evaluate the merit of such speculation, but evidence at hand may be viewed as an intriguing insight into just how closely the bioreactor simulates the microenvironment of developing or remodeling bone.

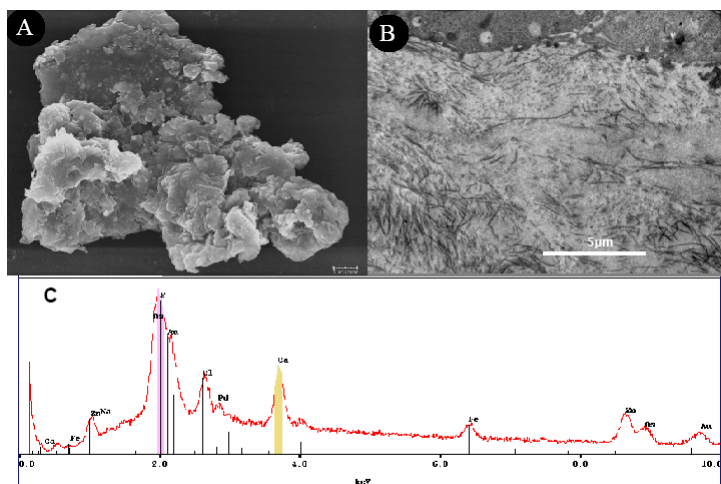


Fig. 2. Analysis of matrix mineralization by MC3T3-E1. Panel A is an SEM of a large nodule taken from a 70 d bioreactor. Panel B is a cross-sectional TEM showing von Kossa stained, mineralized fibers running in and out of the cross-sectional plane. Panel C is an x-ray spectrum obtained by SEM/EDAX of a nodule taken from a 30 d bioreactor confirming Ca and P as major constituents.

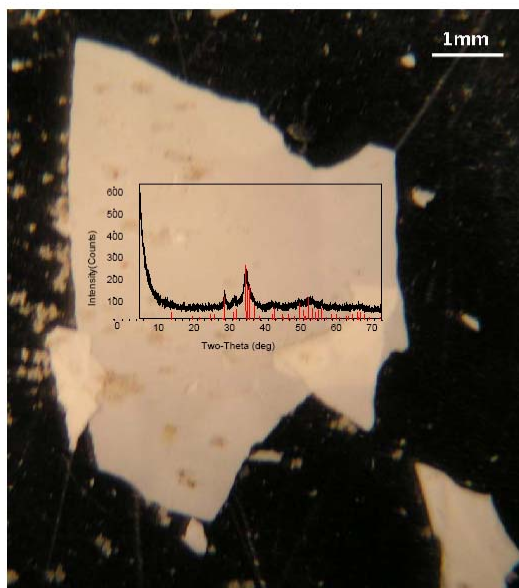


Fig. 3. Bone chip recovered from a 10 month MC3T3-E1 bioreactor. X-ray diffraction annotation indicates similarity with a bovine bone reference spectrum.

We concluded that both the human and the murine osteoblast cell lines can be maintained for indefinite culture periods in the stable environment of the bioreactor wherein a multi-cell-layer, mineralizing tissue grows that eventually develops the characteristic of natural bone.

Significance: Osteoblasts grown in the bioreactor provide a relevant *in vitro* model of natural bone formation suitable for the study of the breast cancer cell colonization of bone.

2. Addition of metastatic breast cancer cells to a MC3T3-E1 bioreactor culture

Main finding: Metastatic breast cancer cells grew in the bioreactor, penetrated the bone-like matrix, and caused the osteoblasts to undergo a change in morphology from cuboidal to elongated.

In a pilot study to determine the interaction of metastatic breast cancer cells with the bioreactor cultures, we added human metastatic breast cancer cells that had been engineered to express GFP (MDA-MB-231^{GFP}) to a 5-month bioreactor culture of MC3T3-E1 cells. The osteoblasts were stained with Cell Tracker Orange™, a vital stain, to allow them to be seen with the fluorescent microscope. We examined the cultures by confocal microscopy every day for three days (Fig. 4).

Fig. 4 compiles the confocal microscopy images of BC penetration of, and replication within, a MC3T3E-1 osteoblast-derived bone matrix. A collection of optical sections (Panel A) corresponding to 1-3 days of BC/bone co-culture indicates that BCs penetrated the entire thickness of the tissue. Panels B-D show 3D reconstructions of optical sections like those shown in Panel A corresponding to days 1-3 of co-culture, respectively. We interpret these images to be multi-layers of MC3T3E-1 incorporated in a thick collagenous matrix (black,) into which columns of BCs penetrated in a few locations during the first day of co-culture (Panel B). Within 2 days of co-culture, BCs replicated (Panel C) and began to organize into linear files especially evident in Panel A, day 3 of Fig. 4. Close inspection of both 2D optical sections and 3D reconstructions revealed concomitant remodeling of the bone-like tissue. Before cancer-cell challenge, osteoblasts exhibited a rounded, cuboidal morphology.³ Over 3 days of BC co-culture, osteoblasts took on a definitively elongated appearance and aligned with cancer cells (Panel A, day 3 of Fig. 4). In particular, it appeared that osteoblasts paralleled the BC files, as though marshaled into an order that seems to further erode the bone-like structure. Similar morphological changes have been observed in osteoblasts exposed to breast-cancer-cell-conditioned medium in conventional culture.^{4,5}

We speculate that tissue destruction occurs through increased apoptosis and the suppression of osteoblast differentiation (as we found to occur in conventional 2D tissue culture^{4,6}), as well as wholesale degradation of the thick extracellular matrix (ECM) in which osteoblasts are embedded (presumably through the action of matrix metalloproteinases and cathepsin K synthesized by cancer cells). Future work will assay the medium for these molecules in effort to confirm this speculation.

Significance: Co-culture of metastatic breast cancer cells with osteoblasts grown in the bioreactor exhibits many characteristics of the metastatic process *in vivo* and mimics the disease process; cancer cell attachment, penetration, colonization, and alignment (filing) were observed.

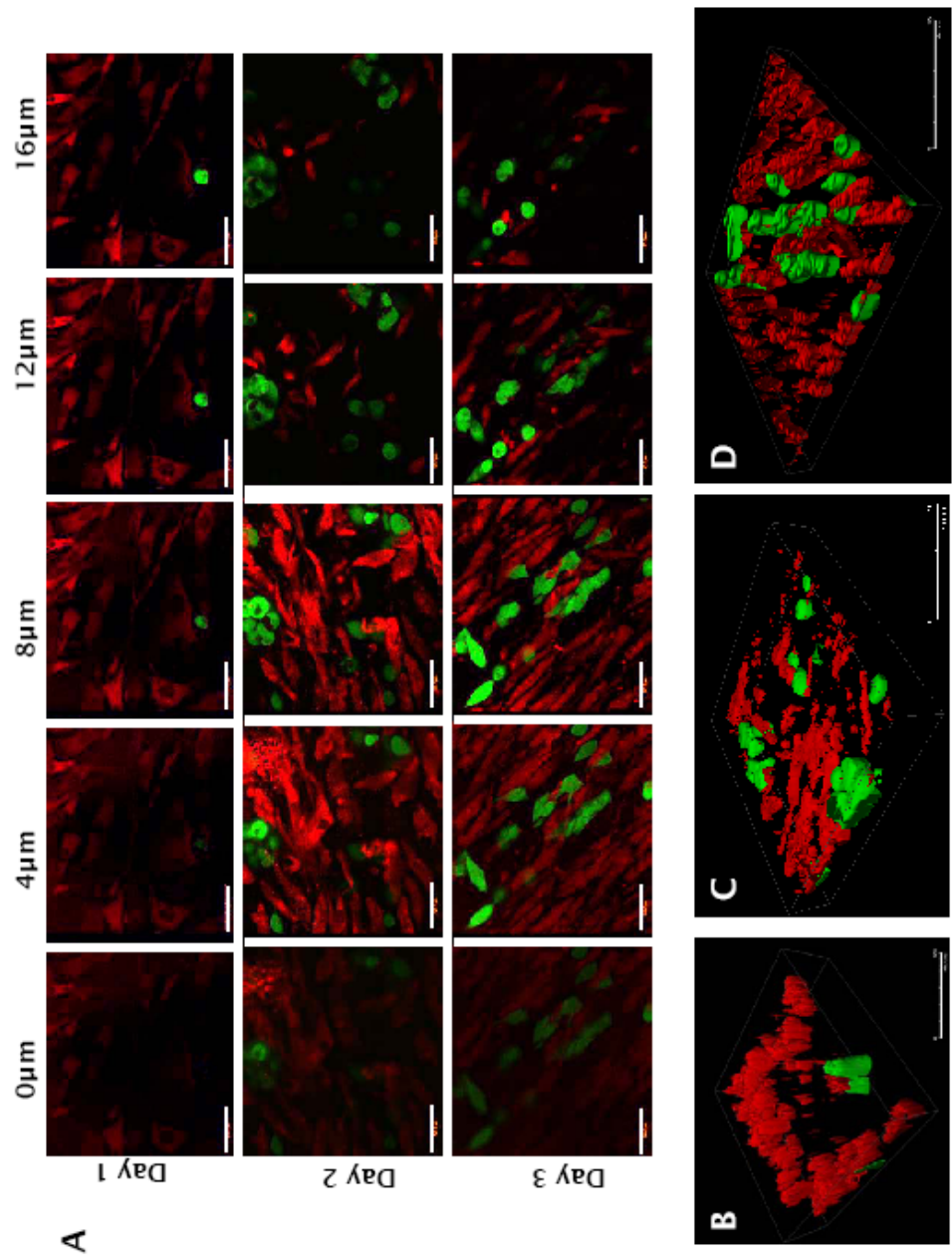


Fig. 4: Serial optical sections through an MC3T3-E1 derived osteoid tissue (5-month culture interval stained with Cell Tracker Orange™) co-cultured with GFP-labeled MDA-MB-231 breast cancer cells (green) for 3 days show that, within 1 day, cancer cells fully penetrate the tissue in a few locations (Panel A, 40X, scale bar = 50 μm). Cancer-cell proliferation (day 2) is followed by organization into a linear-like filling (day 3) with concomitant remodeling of the original osteoid tissue. Optical reconstructions of serial sections over three days (Panels B-D, respectively, 40X) provide a sense of an orchestrated breast-cancer cell attack that ultimately ends with complete destruction of the host osteoid tissue.

3. Breast cancer cell interactions with MC3T3-E1 tissue of different culture ages

Main findings: Osteoblasts underwent a serial phenotypic transition from pre-osteoblasts, to mature cuboidal osteoblasts, to osteocyte-like cells over several months of culture. The interaction of the metastatic breast cancer cells depended on matrix maturity presumably linked to bone cell phenotype.

Based on the observations with the breast cancer cells and the 5-month osteoblast culture, we designed a series of bioreactors to determine the optimal age of the osteoblast culture and ratio of the MDA-MB-231 cancer cells to osteoblasts (Table 1).

Table 1. Design of Co-Culture Combinations of Osteoblasts and Cancer Cells in Bioreactors			
Experimental Parameters	Age of Osteoblasts at the Time of Breast Cancer Cell Inoculation		
Ratio BC:OB	15 days	30 days	60 days
1:10	+	+	+
1:100	+	+	+
1:1000	+	+	+

We used MC3T3-E1 osteoblasts in order to be able to easily distinguish cytokines or other factors secreted by the osteoblasts (murine) from those secreted by the cancer cells (human).

Methods: MC3T3-E1 cells plated at a density of 10^4 cells per cm^2 were cultured in growth medium (α -MEM, 15% FBS and 50 $\mu\text{g}/\text{ml}$ ascorbic acid) until confluent (~a week) and changed to differentiation medium (α -MEM, 10% FBS, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, and 10mM β -glycerophosphate). The medium in the reservoir chamber was changed once every 30 days. After 15, 30 and 60 days of osteoblasts culture, MD-MBA-231^{GFP} human breast cancer cells were added at a ratio of cancer-cells-to-osteoblast of 1:10 (10^5 cancer cells per bioreactor), 1:100 (10^4 cancer cells per bioreactor), and 1:1000 (10^3 cancer cells per bioreactor). Approximately 20% (1.0 ml) of the osteoblast medium was collected for analysis of cytokines prior to cancer cell inoculation. The medium was replaced with the desired number of cancer cells in osteoblast differentiation medium. A Control bioreactor was replaced with differentiation medium without cancer cells. The co-culture was carried on for 7 days. Immediately prior to the addition of cancer cells, the osteoblasts were stained with 10 μM of a vital stain, Cell Tracker OrangeTM (Invitrogen). For fluorescent visualization the light was passed through a 565 nm long pass filter after 543 nm He/Ne excitation. In co-cultures, GFP-expressing cancer cells, excited at 488 nm using an argon laser and collected through 510 nm long pass and 530 nm short pass filters appeared green. The cultures were viewed microscopically over 7 days. On day 7, the bioreactor was dismantled and membrane with intact cells was cut into pieces for various assays (described below). The culture supernatant was collected for cytokine assays. Cell morphology was determined by Laser Confocal Microscopy. By the end of the 7 days the vital stain had faded; therefore, the cells were fixed in 2.5% glutaraldehyde (in cacodylate buffer) and stained for actin filaments with Alexa Flour phalloidin-568 stain (Molecular Probes) and visualized by excitation with a 543 He/Ne laser and collected through a 605 band pass filter. They appeared red. The

light from the GFP-expressing cancer cells was passed through a virtual FV-VC1H filter after excitation at 488 nm using an argon laser. Cell nuclei were stained with DraQ5(data not shown). Images were collected by sequential scans using an Olympus FV300 laser scanning confocal microscope. The Z-stacks were 3D-reconstructed using the software AutoDeblur AutoQuant v9.3. The number of cell layers was determined visually, by counting and following the cells in the 3D reconstructed Z-stack images. IL-6 and OCN in the culture media were determined by ELISA.

Results

3.1. Osteoblast morphology

Main Finding: As osteoblasts proliferated and matured in the bioreactor, morphology gradually changed (as summarized in Fig. 5). Pre-osteoblasts (inoculum) attached and formed multi-layers of cuboidal cells (Fig. 5, 0.7 month) but gradually became more osteocyte-like in appearance (Fig. 5, 1 through 10 months). Osteocyte-like cells were characteristically stellate; the cells exhibited long cytoplasmic processes that extended between other cells and into the bone-like tissue.⁷

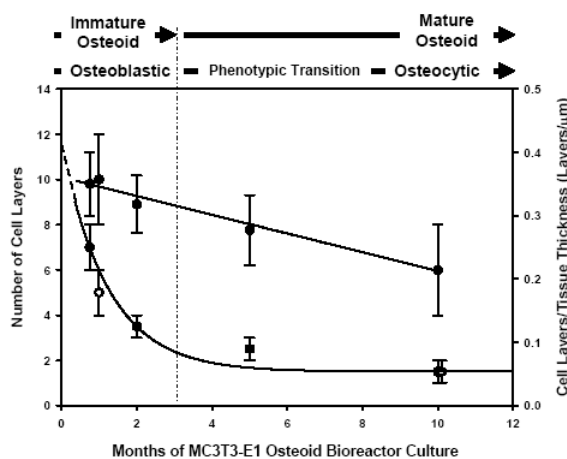


Fig. 5. Summary of significant events observed in bioreactor culture of MC3T3-E1 derived tissue (top) and interaction with MDA-MB-231 breast cancer cells (bottom table).

Initially, within the first month of culture, the number of cell layers increased³ (Fig. 5), but then began to decline as the cells became fewer in number and more osteocyte-like in appearance (Fig. 5). The thickness of the tissue layer also first increased in the first few months but decreased as osteoblast flattened and decreased in numbers (Fig. 5). By 10 months there were approximately 1-2 layers of osteocyte-like cells.

3.2 Co-culture of osteoblasts with MDA-MB-231^{GFP} cells

Main finding: Interaction of the breast cancer cells with osteoblasts in the bioreactor depends on the age of the osteoblast culture and the inoculum of cancer cells.

MDA-MB-231^{GFP} cells were added to 15, 30 or 60-day osteoblast cultures at a BC:OB ratio of 1:10, 1:100 and 1:1000. The osteoblasts were stained with Cell Tracker OrangeTM and the cultures followed by confocal microscopy for 7

days. Results summarized in (Fig. 6) are images from the 1:10 ratio co-cultures.

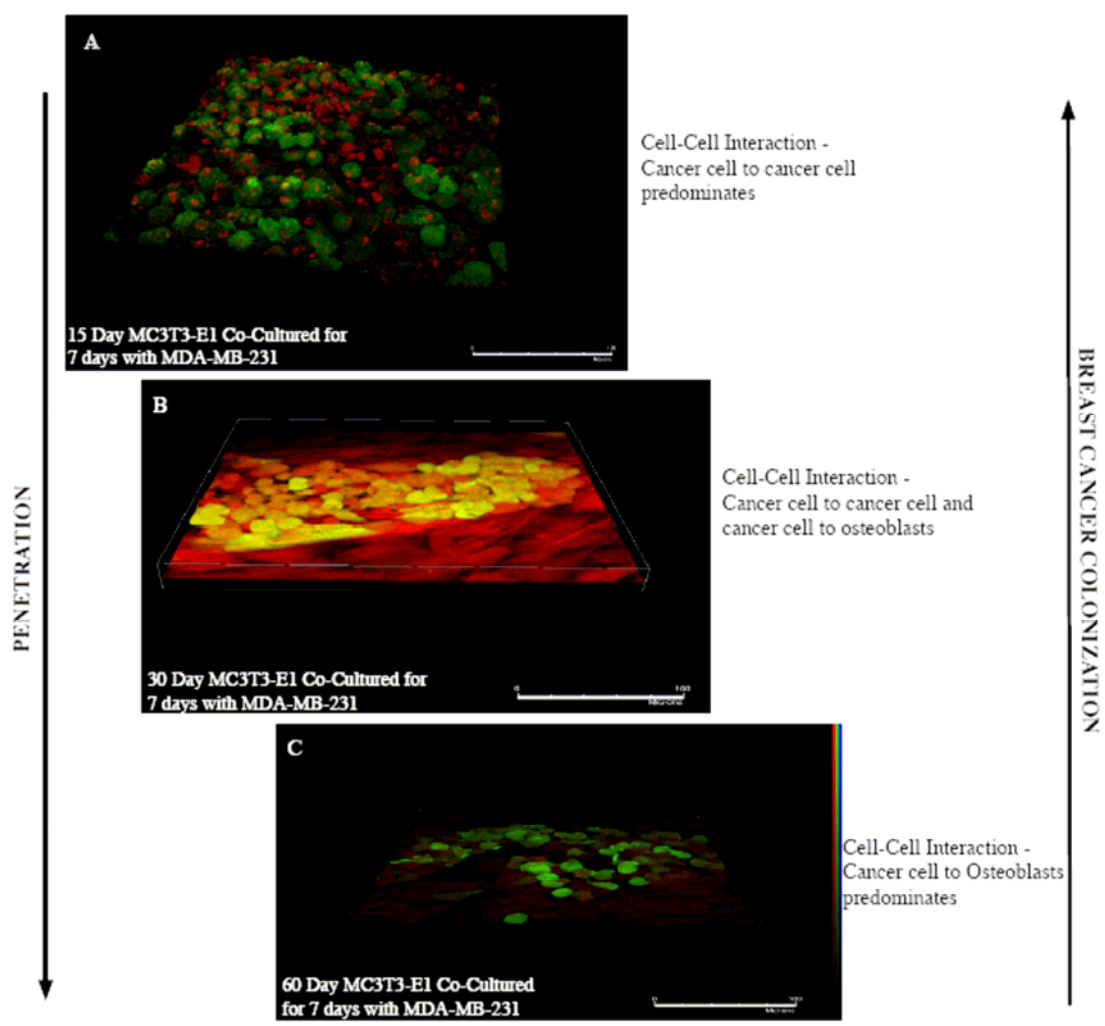


Fig. 6. Growth of metastatic breast cancer cells with bone-like tissue in the bioreactor. MC3T3-E1 osteoblasts were grown for 15 days (A), 30 days (B) or 60 days (C). MDA-MB-231GFP were added at a ratio of ~1 BC:10 OB and monitored by fluorescence microscopy for up to 7 days. Shown are 3 D reconstructions of the images.

At fifteen days, the osteoblasts were early in their differentiation state. The cancer cells (1:10, Fig. 6 A) readily adhered and formed a lawn over the osteoblasts. They did not appear to penetrate the osteoblasts but the osteoblasts were mostly monolayer at this time. We saw a similar growth pattern with cancer cells and osteoblasts grown in tissue culture (see Fig. 12). When breast cancer cells were added to 30-day cultures of osteoblasts, they also attached and grew (Fig. 6 B). They began to penetrate the tissue layer as determined by confocal sectioning of the culture. Some of them were seen to line up and “file” as seen in the 5-month co-culture (Fig. 6 B). Cancer cells also grew into colonies by 7 days. Cultures of 60-day osteoblasts (Fig. 6 C) supported breast cancer cell growth. Some cancer cells penetrated through the tissue. Filing was common and cancer cells formed colonies (tumors), as further illustrated in Fig. 7. The table of Fig. 5 captures significant characteristics of the interaction of metastatic breast

cancer cells with bone-like tissue and relates these interactions with the maturity of the tissue.

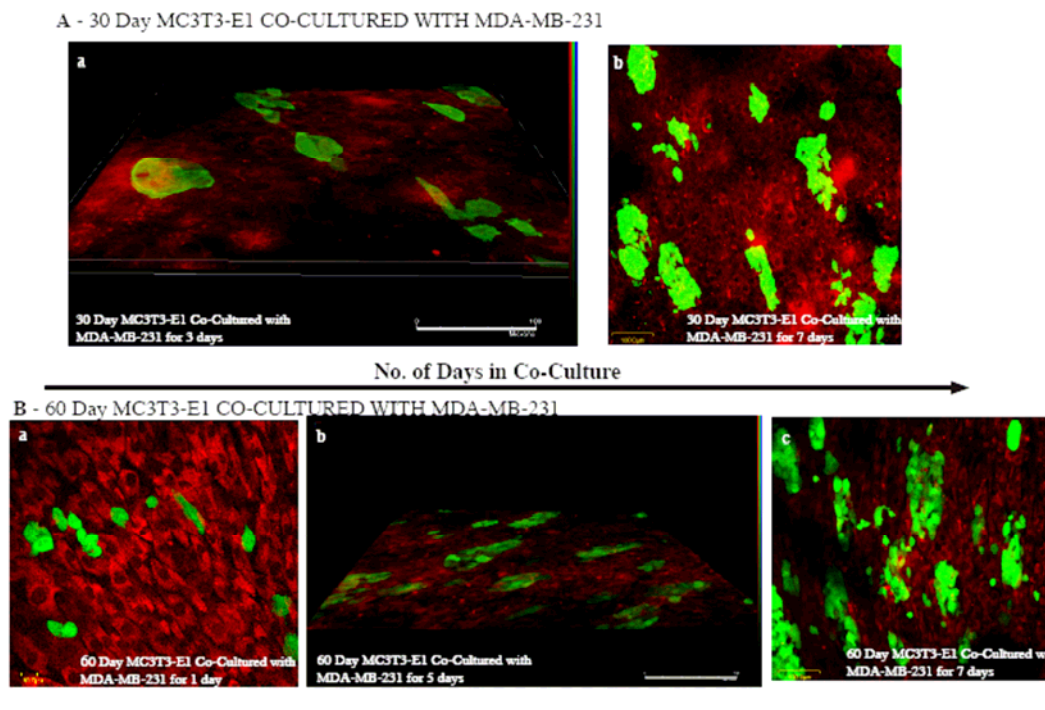


Fig. 7 : Alignment of MDA-MB-231^{GFP} metastatic breast cancer cells co-cultured with MCA3T3-E1 osteoblasts. Cancer cells were inoculated at about 1 BC:10 OB at 30 (A) or 60 (B) days of osteoblast culture and examined by confocal microscopy each day for 7 days. Shown are the confocal images of the co-cultures. A=3 and 7 days co-culture. B= 1,5 and 7

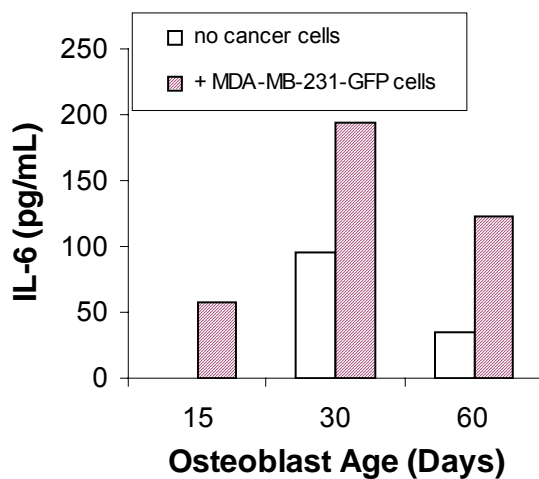


Fig. 8: Induction of osteoblast IL-6 by MDA-MB-231 cells. MDA-MD-231^{GFP} cells were added to bioreactors of MC3T3-E1 cells that had been cultured for 15, 30 or 60 days. A sample of culture medium was collected before addition of the cancer cells and again after 7 days. The media were assayed for IL-6 with ELISA.

Cancer cell interaction with bone-like tissue caused the osteoblasts to decrease production of IL-6 (Fig. 9), a characteristic osteoblast inflammatory stress cytokine. We also found that osteocalcin (OCN) was present in the culture medium (Fig. 9). OCN is a secreted molecule characteristic of mature osteoblasts. In our previous work we found that OCN expression was reduced in osteoblasts grown in standard tissue culture in the presence of breast cancer cell conditioned medium.⁸ In the bioreactor system, we found

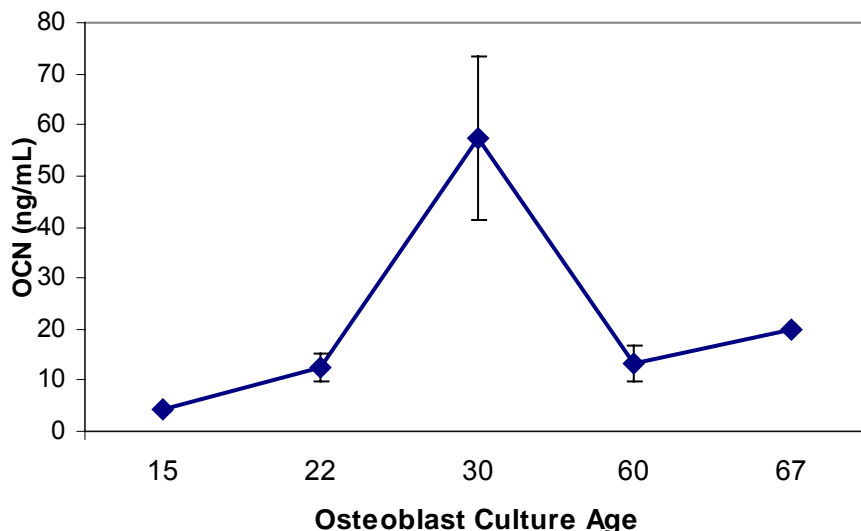


Fig. 9: Production of OCN by MC3T3-E1 cells grown in the bioreactor for the indicated number of days.

that OCN production peaked at day 30 in the absence of cancer cells (Fig. 9).¹ Preliminary data obtained in the presence of cancer cells suggests that the peak in OCN production was subdued (Fig. 10).

Significance: It is clear that maturity of the bone tissue significantly influences the nature of interaction

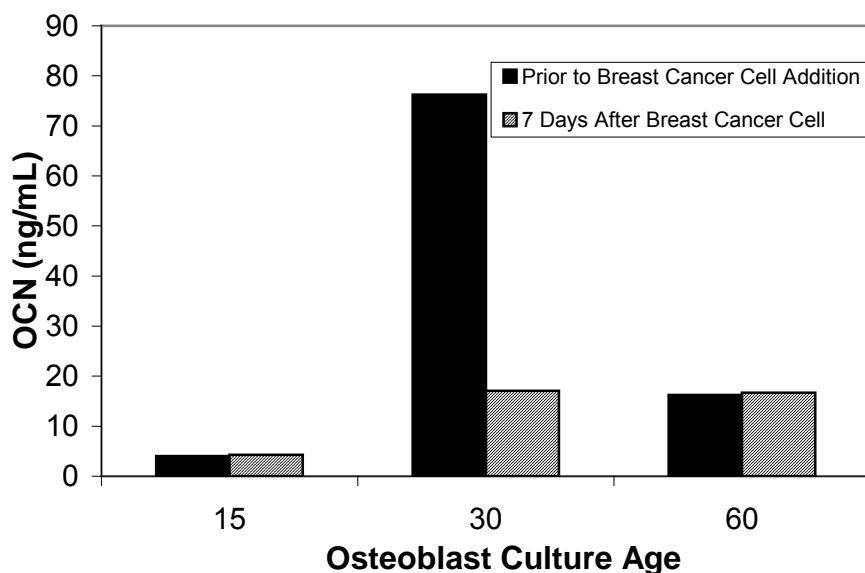


Fig. 10. Osteoblasts in the presence of MDA-MB-231 cells at a ratio of 1:10 for 7 days. A sample of the culture medium was collected before addition of the cancer cells and after 7 days of co-culture.

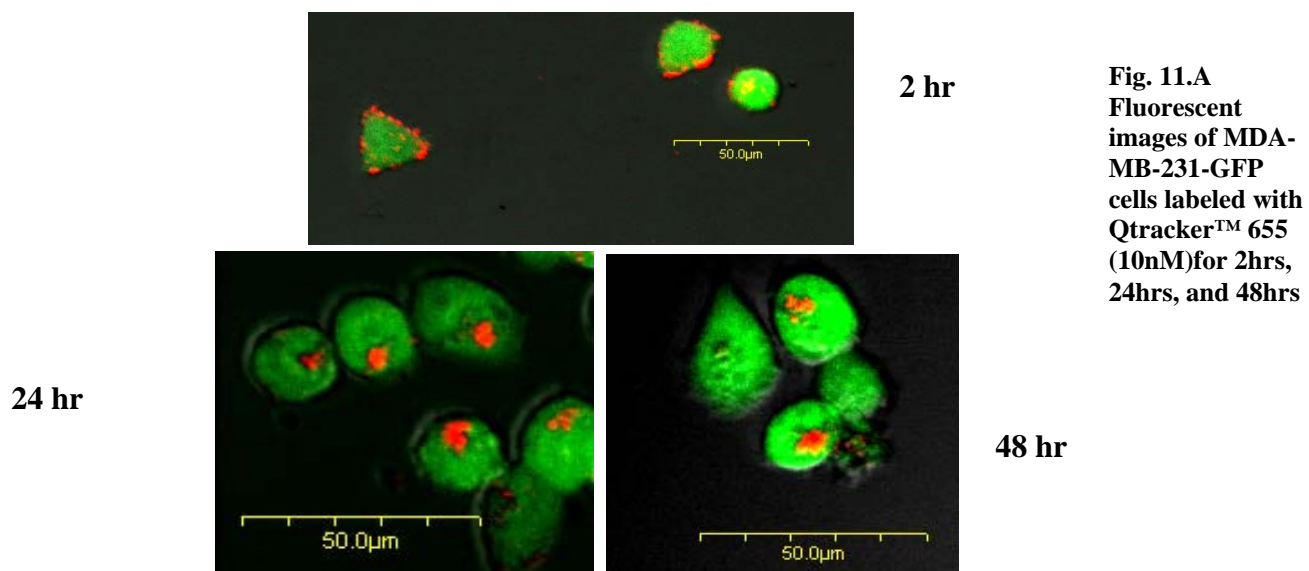
¹ OCN values cannot, at this time, be interpreted on an absolute basis because it is a small molecule (5.8KD) partially removed in monthly medium changes. Future work we will determine mRNA expression of OCN and other characteristic osteoblast molecules.

between breast cancer cells and osteoblasts. By selecting the appropriate culture age, we can compare and contrast interaction of cancer cells with a full range of bone tissue maturity (Fig. 15) to better understand the molecular and cellular basis of breast-cancer cell colonization of bone.

3.3. The use of Q-Dots to detect the interaction between osteoblasts and breast cancer cells in the bioreactor

Main Finding: Cancer cells can be labeled with electron dense Q-Dots for identification at the TEM level and can be distinguished from osteoblasts.

The use of GFP-expressing cancer cell lines has provided a powerful method to view the interaction of the cancer cells with osteoblasts at the fluorescent microscope level. However, at the TEM level (which is necessary for ultra-structural analysis), fluorescence is lost. This property greatly complicates secure differentiation of osteoblast from breast cancer cells. As an alternative to fluorescence-based cell differentiation, we have explored the use of Q-dot cell labels. Q-Dots are a convenient source of electron-dense nanoparticles that are readily taken up by cells and can thus serve as a TEM marker.



We first carried out experiments in culture slide chambers to establish conditions before we committed the time and resources to a bioreactor. We found that the cancer cells readily took up the nano-particles (Fig. 11 A). The particles did not affect cell viability or growth over eleven days (data not shown). Qdot labeled MDA-MB-231^{GFP} cells were co-cultured with MCA3T3-E1 cells grown in slide cultures. After 24 hours the cultures were prepared for TEM (Fig. 11B). The breast cancer cells showed processes of thickened membrane adjacent to the osteoblasts. The two cell types joined by cilia-lie structures. We now plan to study the cell-cell interactions in more detail in a bioreactor.

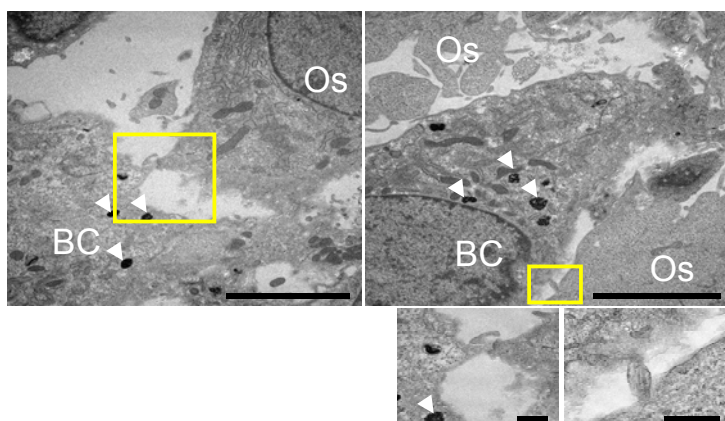


Fig. 11. TEM images of Qdots-labeled breast cancer cells (BC) and osteoblasts (Os) 24 hrs after co-culture. Breast cancer cells were identified by the Qdots found in the intracellular compartments (arrows). Breast cancer cells interacted with osteoblasts through processes of thickening membrane (upper panels). Occasionally, cilia-like structure were seen to bridge the cancer and bone cells (lower panels). The lower panels are images of high magnification of selected areas in the upper panels. Bars = 5 μ m in a and b; 0.5 μ m in c.

4. Co-culture of osteoblasts and breast cancer cells in standard tissue culture

Main Finding: Osteoblastic tissue derived from conventional culture is not the optimal model for studying cancer-cell colonization of bone.

For comparison with bioreactors and to examine early co-culture with early cultures of osteoblasts, we conducted a series of experiments in which MC3T3-E1 cells were cultured in standard polystyrene tissue culture plates. MDA-MB-231^{GFP} cells were added at the same ratios as in the bioreactor 1:10, 1:100 and 1:1000. The osteoblasts were cultured for 4 days (proliferation), 14 days

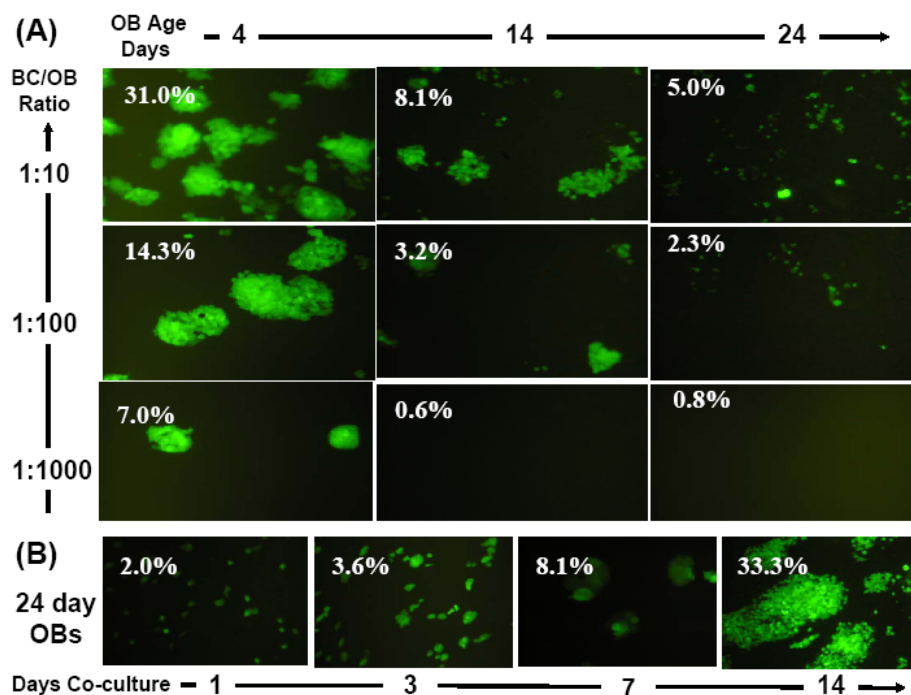


Fig.12. Fluorescence microscopy of osteoblasts co-cultured with GFP-secreting BCs using conventional culture methods in which BCs fluoresce green against a black osteoblast layer(s) background. Panel A shows the effect of increasing osteoblast maturity and estimated BC/osteoblast cell-number ratio. Panel B shows the effect of increasing co-culture interval at fixed 1:10 BC/Ob cell number. Percent coverage by BCs was estimated using image analysis (Image J, NIH) and is annotated in the upper-left corner of each image.

(early differentiation) and 24 days (late differentiation) before the cancer cells were added. Cultures were stopped after 1, 3 or 7 days of co-culture (Fig.12, panel A). The cultures were followed daily by fluorescence microscopy. The size of the cancer colonies was computed from several areas on the plate by the Image J (Fig.12, panel A). The percent area of the culture covered by cancer cells was calculated.

We also determined the effect of inoculating a well-differentiated osteoblast culture (24 days) with breast cancer cells at a ratio of 1:10 for as long as 14 days (Fig.12, panel B). By 7 days the cancer cells had not grown to a great extent but by 14 days they covered >30% of the osteoblast culture, equivalent to that seen in just a few days with proliferating osteoblasts (Fig. 12, panel A, 1:10 vs. 4 days). Graphical analysis of the optical data of Fig. 12 revealed that the cancer cells grew at different rates depending both on the size of the inoculum and on the age of the osteoblasts (Fig.13). However, when the osteoblasts are most differentiated in culture the growth of the cancer cells was approximately the same regardless of the initial inoculum.

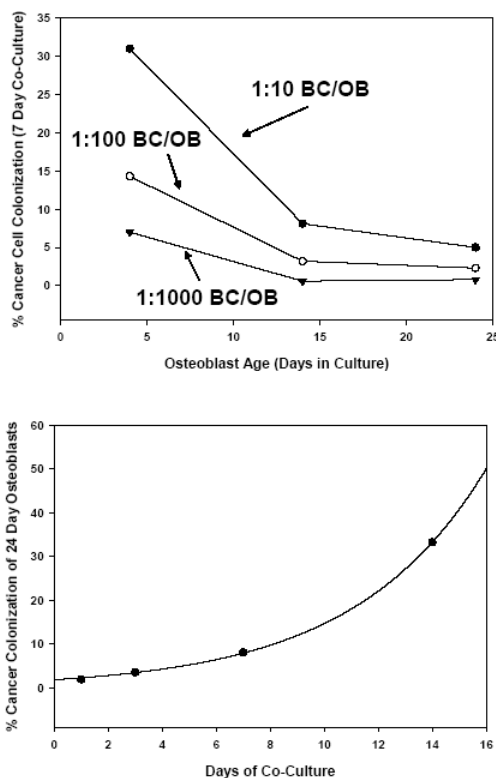


Fig. 13. Quantitative image analysis of Fig. 11 showing (Panel A) that BCs colonize relatively immature OBs in culture more rapidly than more mature ones; and (Panel B) exponential growth of BCs on mature in conventional culture.

We also assayed the culture media for IL-6 (Fig.14). At every culture combination tested, the addition of cancer cells to the osteoblasts induced expression of osteoblast IL-6. The extent of the increase varied. With osteoblasts grown for 4 days at the time of the addition of breast cancer cells, the expression of IL-6 was induced by the greatest amount after 7 days (16.5 fold). At every other time there was less of an increase. However, standard tissue culture requires that the medium be replaced every 2 to 3 days. Thus, IL-6 would have been removed with the medium changes.

Significance: Standard tissue culture can be useful for studying short-term interactions. However, we did not observe cancer cell alignment or penetration that is characteristic of pathological tissue. Therefore,

we conclude that three-dimensional tissue models that can be sustained long-term in culture are essential to mimicking cancer cell colonization of bone *in vitro*.

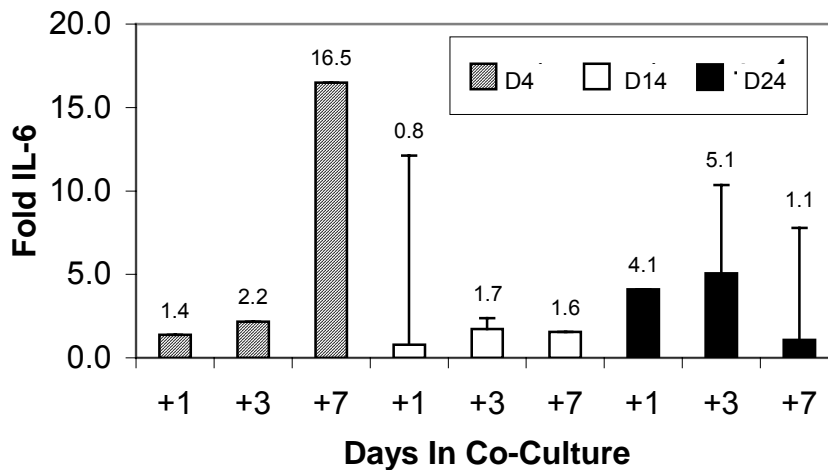


Fig. 14. Fold induction of IL-6 secreted from osteoblasts in the presence of metastatic breast cancer cells. Metastatic breast cancer cells were added to osteoblasts grown in tissue culture for 4, 14, or 24 days. The cells were in co-culture for 1, 3, or 7 days before culture media was collected and tested for the presence of IL-6 by ELISA. N=1 for D4, n=2 for D14, and n=3 for D24.

5. Osteocytic Transformation

Major finding:

Osteoblasts undergo a phenotypic transformation to osteocytes.

We maintained one culture for approximately 10 months. At the end of that time we saw a macroscopic bone chip (Fig. 3) as well as deposition of mineral on the dialysis

membrane. When the bioreactor was disassembled, part of the membrane was fixed and the cells were visualized by confocal microscopy, TEM, and SEM (Fig. 5) revealing that cells exhibited characteristics of osteocytes,⁹ especially stellate shape and long inter-cellular processes. Cells from another portion of the membrane were lysed and the RNA isolated. This material will be used to carry out quantitative RT-PCR for the presence of various characteristic osteoblast and osteocyte proteins.

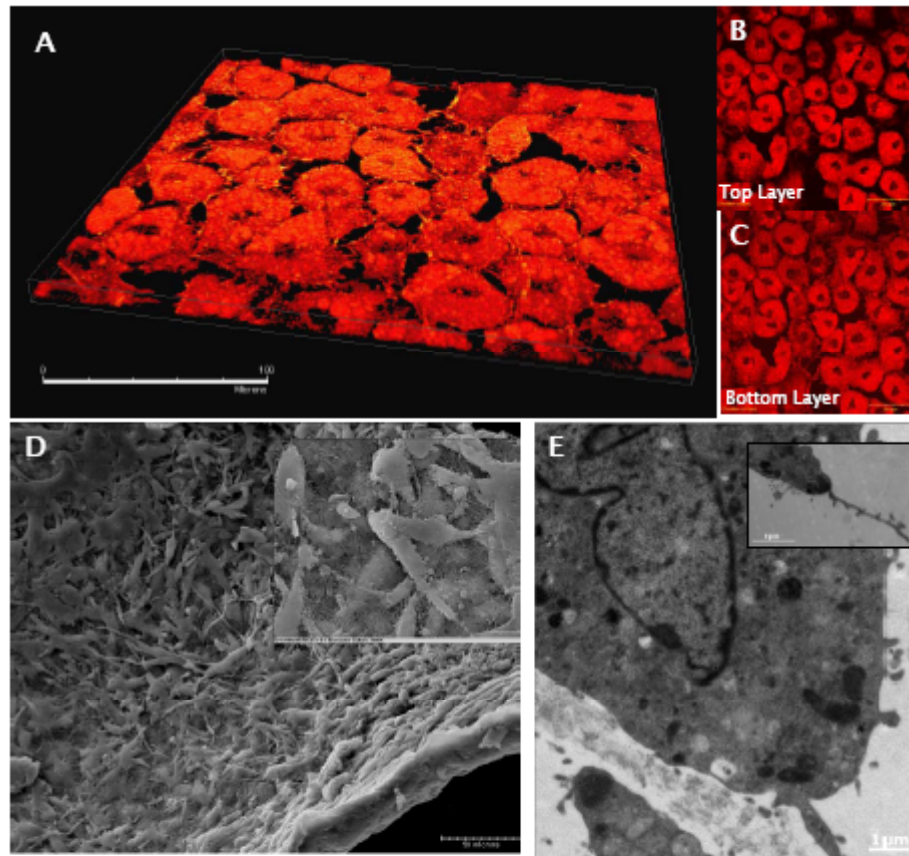
Significance: Osteocytes are terminally differentiated cells that are surrounded by mineralized matrix *in vivo*. It is very difficult to isolate and grow these cells in culture. There are only a few lines with osteocyte characteristics. Thus it is significant that we were able to grow these cells in the bioreactor. In addition, by selecting the appropriate culture time, we can see a progression of cells from osteoblast to osteocyte; i.e. the upper layer contains osteoblasts while the bottom layer contains osteocyte-like cells.

Task 3: To test known stimulators and/or protectors of osteoblast function in the presence and absence of breast cancer cells in order to develop a means of blocking the destructive effects of breast cancer cells have on bone forming osteoblasts. (months 14-34).

Task 3 has not yet been carried out.

Establish cultures of osteoblasts at various stages of differentiation in the presence and absence of metastatic breast cancer cells as determined from task 1. Neutralizing antibodies to TGF-beta will be added and the cultures followed as in task 1.

Establish cultures of osteoblasts at various stages of differentiation in the presence and absence of metastatic breast cancer cells as determined from task 1. Glutamine will be added and the cultures followed as in task 1.



- A: 3-D reconstruction of multilayered MC3T3-E1 tissue after 10 months of bioreactor culture (60X).
- B, C: Top and Bottom Layers of tissue shown in 4.A (60X)
- D: S.E.M of the surface of a 10 month culture (350X). Inset shows detail of the same the same culture (1500X)
- E: Transmission electron micrograph section of a 10 month culture (4000X). Inset :TEM section of the same culture showing extended cellular process (4000X)

Fig. 15. Morphology of MC3T3-E1 cultures after 10 months of culture in the bioreactor.

Establish cultures of osteoblasts at various stages of differentiation in the presence and absence of metastatic breast cancer cells as determined from task 1. Selenium will be

increased or reduced (by used of selenium depleted serum) and the cultures will be followed as described in task 1.

ending on the outcomes of a-c, combinations of these compounds will be added to the cultures. Osteoblasts will be assayed as described in Task 1.

Task 4: To analyze the data and write manuscripts. (Months 35-36)

We have been analyzing the data as it is obtained. One manuscript on the initial characterization of the osteoblasts grown in the bioreactor has been published (Dhurjati R., Liu X., Gay C.V., A.M. Mastro and Vogler E.A. Extended-Term Culture of Bone Cells in a Compartmentalized Bioreactor. 2006. Tissue Engineering. 12: 3045-3054).

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstrated that a specialized bioreactor could be used to grow and maintain osteoblasts for extended times, at least 10 months, in culture.
2. Characterized the osteoblast cultures over time in the bioreactor.
3. Determined that the osteoblasts grew in multilayer and formed a mineralizing matrix.
4. Discovered that the osteoblasts transitioned to become osteocyte-like cells with time in culture.
5. Discovered that metastatic breast cancer cells in co-culture with the osteoblast tissue exhibited many of the characteristics of metastases-bone interactions described *in vivo*.
6. The cancer cells attached to the osteoblast bone-like tissue and grew.
7. Depending on the culture age and cancer cell inoculum, the cancer cells aligned themselves into columns (filing). At later stages, they formed colonies of cells from these columns.
8. The cancer cells penetrated the bone-like tissue and caused loss of matrix.
9. The osteoblasts changed shape from cuboidal to elongated and spindle shaped, in alignment with the cancer cells.
10. The osteoblasts increased expression of an inflammatory stress response marker, IL-6.

REPORTABLE OUTCOMES

Manuscripts, Publication

Dhurjati R., Liu X., Gay C.V., A.M. Mastro and Vogler E.A. Extended-Term Culture of Bone Cells in a Compartmentalized Bioreactor. 2006. Tissue Engineering. 12: 3045-3054.

Abstracts and Presentations

1. Laurie A. Shuman, Ravi Dhurjati, X. Lui, Carol V. Gay, Erwin A. Vogler, and Andrea M. Mastro. 2006. A New *In Vitro* Model of Breast Cancer Metastasis to

- Bone. 97th Annual Meeting of the American Association for Cancer Research, Proceedings. 47: 267.
2. Ravi Dhurjati, Laurie A. Shuman, Carol V. Gay, Andrea M. Mastro, and Erwin A. Vogler. 2006. Indefinite-Term Culture of Bone Cells in a Compartmentalized Bioreactor. Pennsylvania State University Regenerate World Congress on Tissue Engineering and Regenerative Medicine, April 25 - 27, 2006, Pittsburgh, Pennsylvania.
 3. Ravi Dhurjati, Laurie A. Shuman, Carol V. Gay, Andrea M. Mastro, and Erwin A. Vogler. 2006. Compartmentalized Bioreactor for Long-term Culture of Bone Cells. Pennsylvania State University Annual Meeting and Exposition of the Society for Biomaterials, April 26 - 29, 2006, Pittsburgh Pennsylvania.
 4. Andrea M. Mastro, Laurie S. Shuman, Ravi Dhurjati, X. Lui, Carol V. Gay and Erwin A. Vogler. A New *In Vitro* Model of Breast Cancer Metastasis in Bone. The 11th Congress of the Metastasis Research Society, September 3-6, 2006 in Tokushima, Japan. Abstract PD6-8 p 119.
 5. Ravi Dhurjati, Laurie A. Shuman, Vekatesh Krishnan, Andrea M. Mastro, Carol V. Gay, and Erwin A. Vogler. Compartmentalized Bioreactor: *In Vitro* Model for Osteobiology and Osteopathology. 28th Annual Meeting of the American Society for Bone and Mineral Research, September 15-19, 2006 in Philadelphia, PA. Vol. 21, Suppl 1, ps 349 #MO87.
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 9. Laurie A. Shuman, Ravi Dhurjati, Vekatesh Krishnan, Gang Ning, Erwin A. Vogler, Carol V. Gay Andrea M. Mastro. Use of Qtracker™ 655 to monitor the interaction of metastatic breast cancer cells with osteoblasts in co-culture. Proceedings of the 98th Annual Meeting of The American Association for Cancer Research, Los Angeles, CA, April 2007. vol 48.
 10. Karen M. Bussard and Andrea M. Mastro. "Osteoblast-Derived Inflammatory Cytokines are Produced in Response to Human Metastatic Breast Cancer Cells." Proceedings of the 98th Annual American Association for Cancer Research Conference, Los Angeles, CA. April 2007, vol 48.

CONCLUSIONS

The use of a specialized bioreactor has allowed us to grow osteoblasts into a bone-like tissue over an extended time. The resulting 3D mineralizing tissue can be challenged with metastatic cancer cells creating a system “in crisis” that simulates early stages of metastatic breast cancer colonization of bone. This model will allow *in vitro* testing of drugs and other therapeutics in a system that far surpasses standard tissue culture.

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APPENDIX

Dhurjati R., Liu X., Gay C.V., A.M. Mastro and Vogler E.A. Extended-Term Culture of Bone Cells in a Compartmentalized Bioreactor. 2006. Tissue Engineering. 12: 3045-3054.